1. The proposed mechanism of different actions of alcalase and trypsin during protein fragmentation

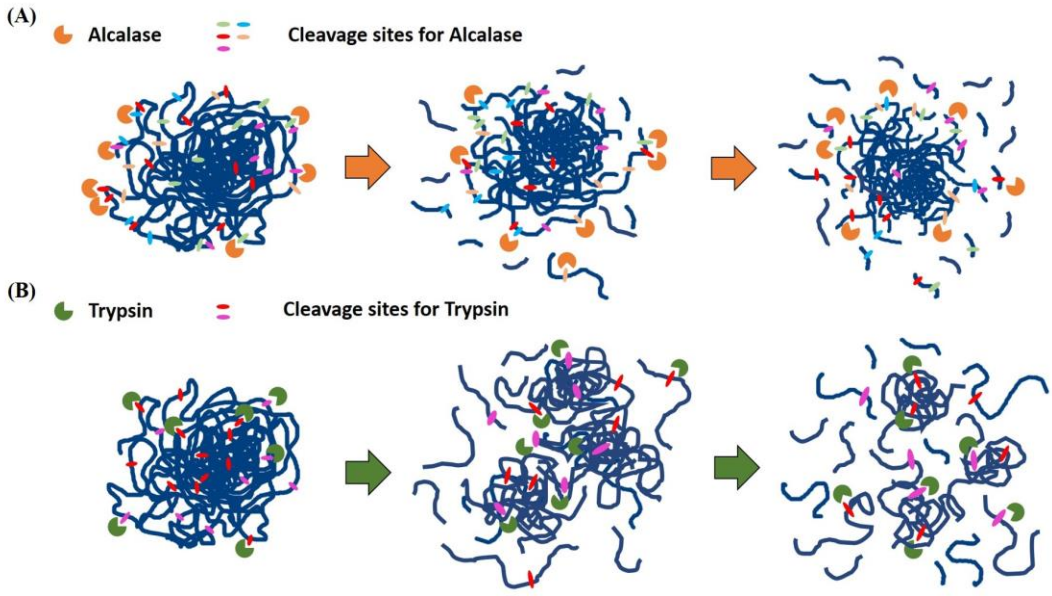

Fig.S1 The schematic picture of the processes of protein hydrolysis by alcalase (A) and trypsin (B).

As discussed in section 3.1, alcalase is assumed to predominantly hydrolyse protein chains residing close to the surface of the protein aggregates, due to its low selectivity and large choice of peptide bonds to break. It therefore produces a large number of small protein fragments with less affected core of protein aggregates simultaneously remaining. In contrast, due to the high selectivity, trypsin needs to be able to get deeper into the core of protein aggregates to find suitable peptide bonds it can cleave, if it is to achieve the same degree of hydrolysis. This may take longer, but it will tend to fragment chains throughout the whole body of the aggregated protein particles. This means that trypsin is more effective than alcalase in reducing the protein particle size. Also, the peptides obtained by trypsin hydrolysis tend to be of intermediate size.

2. The molecular weight profiles of various whey protein samples

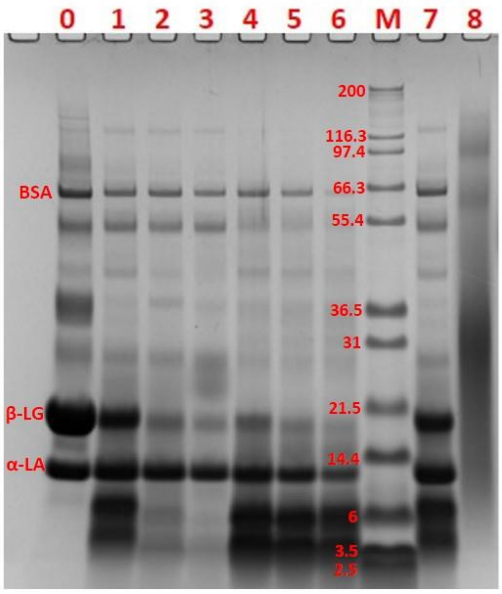

Fig.S2 Reducing SDS-PAGE analysis of the protein/peptide profiles for various whey protein samples. Lane 0 is intact WPI. Lane 1-3 are polypeptides produced by trypsin digestion at increasingly higher DH (i.e. WT1 at 2.5%, WT2 at 5.5% and WT3 at 8.0%, respectively). Lane 4-6 are polypeptides produced by alcalase digestion, from lower to higher DH (i.e. WA1 at 2.5%, WA2 at 5.5% and WA3 at 8.0%, respectively). Lane M is the molecular weight ladder. A sample post conjugation with maltodextrin (i.e. WT1-MD) is also shown at lane 8 to compare with its unconjugated counterpart (i.e. WT1) in lane 7.

The major components of intact WPI, i.e. β-LG, α-LA and BSA, were marked on the gel sheet (lane 0). The component α-LA showed more resistance to enzymatic attack than β-LG. It is clearly seen that trypsin and alcalase generated polypeptides with distinct profiles. We find trypsin gradually broke whey protein down. This is seen as a shift of bands towards lower molecular weight range with increasing DH (lane 1-3). As for alcalase, the profiles of hydrolysates (lane 4-6) did not show a distinct difference with increasing DH beyond 2.5%.

The successful formation of conjugates was confirmed as well. The presented results here are limited to conjugates formed using WT1, though similar data (not shown) were also obtained for other hydrolysed samples. In comparison to the equivalent unmodified protein fragments (lane 7), a noticeable shift in molecular weight, towards higher values, was observed for conjugated WT1 (lane

8). This increase in molecular weight is indicative of the formation of covalent bonds between maltodextrin (M_w= 8.7 kDa) with our protein fragments.

3. The solubility of WPI and WPHs samples

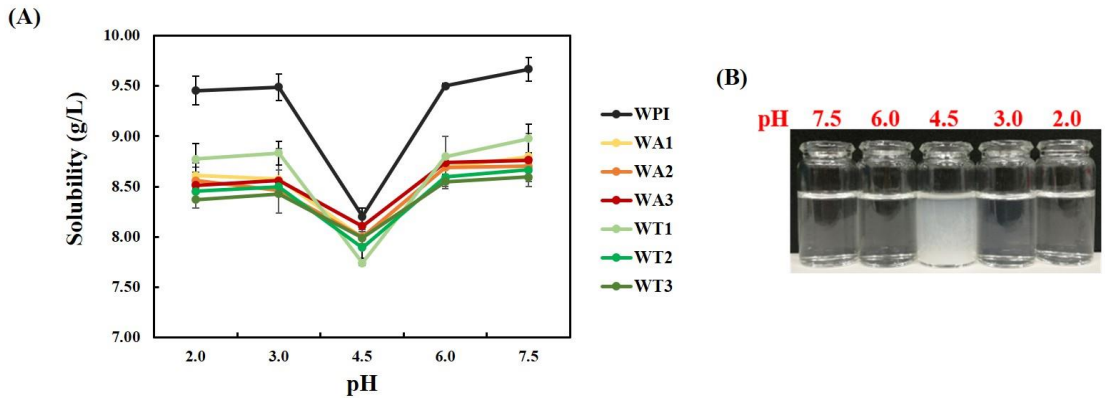

Fig.S3 (A) Solubility of intact WPI and WPHs samples hydrolysed by trypsin and alcalase at various DH, plotted as a function of pH. (B) The visual appearance of 1% (w/v) WT1 sample solution at various pH conditions.

Starting with the intact WPI as the first example, the above results showed that the protein has the lowest solubility at pH 4.5, which indeed is at its reported pI value as expected. At all other pH conditions (pH 7.5, 6.0, 3.0 and 2.0), WPI exhibited a high level of solubility. The same trend was observed for all the hydrolysed whey protein samples produced under the action of either enzyme (WT1, WT2, WT3 and WA1, WA2, WA3).

The change in the solubility with pH was also seen visually in **(B)**. The figure displays the variation of the solubility of 1% (w/v) WT1 solution as a function of pH. A clear solution was obtained at all pH values except at pH 4.5. At pH = 4.5, the fragments aggregated and settled down due to their reduced average charge.

This figure also showed a distinct drop in the solubility for intact WPI post its limited hydrolysis of up to DH 8.0%, by either trypsin or alcalase. This decrease was seen at all tested pH conditions.

4. The solubility of conjugated WPI and WPHs samples

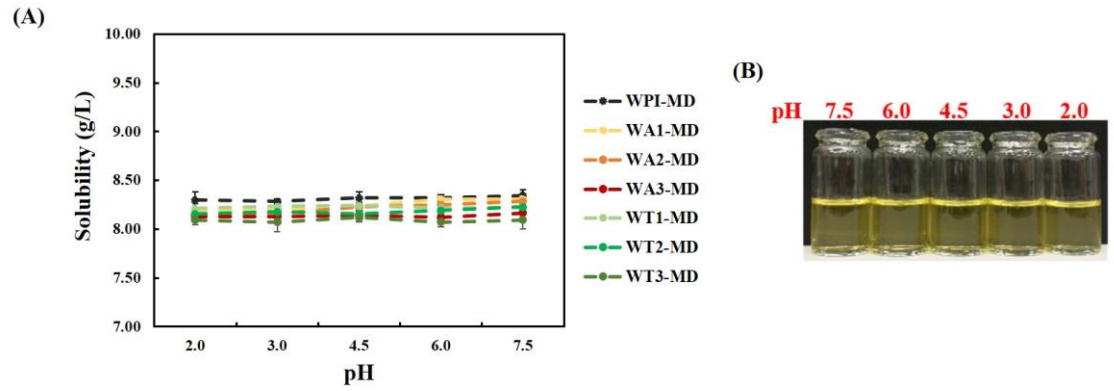

Fig.S4 (A) Solubility of conjugates made from intact WPI and those from WPHs at various levels of hydrolysis, produced by either trypsin or alcalase (i.e. WT1-MD, WT2-MD, WT3-MD and WA1-MD, WA2-MD,WA3-MD samples). Solubility is plotted as a function of pH. (B) The visual appearance of 1% (w/v) WT1-MD sample solution at various pH conditions.

The solubility profiles of conjugated samples generally displayed rather flat lines without any significant changes across the studied pH range. This is in contrast to the large variation seen in the solubility of unconjugated samples with changing pH (**Fig.S3**).

The Maillard reaction products, formed from covalent bonding of WPI or WPHs with maltodextrin, showed a typical golden-brown colour. When dissolved in deionised water, they formed a clear golden solution at all tested pH conditions, including pI of WPI, without formation of any visible aggregates. The visual appearance of the solution involving sample WT1-MD is shown in **(B)**. This improvement in solubility at pI was also quantitively seen (compare **Fig.S3** and **Fig.S4**). For example, the solubility of WT1 post conjugation with maltodextrin increased to 8.2 g/L from 7.7 g/L for unreacted WT1 sample. Nonetheless, at conditions away from pI, we noticed a slight decrease in the solubility of the conjugates in comparison to non-reacted equivalents (compare **Fig.S3** and **Fig.S4**). For instance, the solubility of WT1 at pH conditions other than 4.5 was measured to be around 8.8 g/L, while that of WT1-MD was approximately 8.2 g/L at the corresponding pH conditions. The possible mechanism for causing the reduction of solubility post Maillard reaction is discussed in section 3.3.2.

5. Morphology and stability of emulsions at acidic pH conditions

5.1 Emulsions based on WPI and WPHs samples

At neutral pH conditions, WPI is well known to be able to form fine stable emulsions, as is also observed in **Fig.6B**. The droplet size, $D_{4,3}$, of fresh emulsion stabilized by WPI at pH 7.5 was 0.682 μm, although this increased to 0.833 μm after 60 days.

The hydrolysis by trypsin at DH 2.5% (WT1) produced fragments with improved emulsifying and stabilizing capacities compared to the intact WPI (**Fig.6B**). The micrograph of fresh WT1 stabilized emulsion at pH 7.5 (**Fig.7B**), showed fine oil droplets ($D_{4,3}$ = 0.628 μm) with a monomodal size distribution. The ζ-potential was measured to be around -55.7 mV (**Table 1**), indicating the presence of strong electrostatic repulsion between the droplets. The emulsion remained reasonably stable, with $D_{4,3}$ = 0.656 μm, even after 60 days (**Fig.7B**). However, as WPI was further fragmented to achieve higher DH values of 5.5% and 8.0% (WT2 and WT3), the emulsifying functionality was found to suffer. The droplet sizes at day 1 were 0.837 μm and 1.49 μm for emulsions made by WT2 and WT3, respectively. These grew to 1.37 μm and 1.73 μm after 60 days of storage at pH 7.5 (**Fig.6B**).

As for fragments produced by alcalase digestion, no improvement was observed. Both the emulsifying and stabilizing properties of WPI worsened from the very onset as a result of hydrolysis (**Fig.6B**). The droplet sizes, $D_{4,3}$ of fresh emulsions made by alcalase generated WPHs at day 1 were 0.713 μm (WA1), 1.02 μm (WA2) and 1.56 μm (WA3), which were noticeably ($p < 0.05$) larger than the ones made with the intact WPI ($D_{4,3}$ = 0.682 μm). After 60 days of storage, these values increased to 1.65 μm, 2.16 μm and 3.02 μm for WA1, WA2 and WA3 stabilized emulsions, respectively. This was to be compared to 0.833 μm found for the unmodified WPI at day 60 (**Fig.6B**). Nonetheless, the possibility that at even lower DH values of less than 2.5%, a small improvement may be possible cannot be entirely ruled out. While we did not investigate this point further, if observed it will confirm a similar trend as that found for trypsin produced WPHs. That is to say, a modest improvement at low levels of fragmentation is followed by inferior emulsifying properties as the protein is broken down even more.

As seen above, the overall differences between the performances of WPI derived polypeptides produced by trypsin and alcalase, at currently investigated levels of DH, are relatively small.

When the pH of the whey protein based emulsions (i.e. those made from the intact WPI or WPHs produced by digestion with trypsin and alcalase) was adjusted to pH 4.5, the originally well dispersed droplets became strongly destabilized. This was illustrated by the dramatic increase in droplet size, as indicated in **Fig.6B**. For example, the measured $D_{4,3}$ rose from 0.628 μm at pH 7.5 to 11.1 μm at pH 4.5, for fresh WT1 stabilized emulsion. This is attributed to the reduced electrostatic stabilization (ζ-potential = +11.1 ± 0.6 mV, see **Table 1**). The micrograph of fresh WT1 stabilized emulsion at pH 4.5 displayed the formation of clusters of highly flocculated droplets (**Fig.7B**). We also observed a significant increase in the low shear viscosity of the emulsion compared to that at pH 7.5 (**Fig.S6** in supplementary). After 60 days of storage, a few large droplets were observed in the micrograph of WT1 stabilized emulsion stored at pH 4.5 (**Fig.7B**).

If the pH was subsequently lowered down to 3.0, reasonably quickly following its previous adjustment to pH 4.5, the flocculated droplets were seen to redisperse to a large extent. This observation was true of WPI and all WPHs based samples. It can be seen from the much smaller size measured following such an adjustment of pH, as indicated by the average sizes $D_{4,3}$ at pH = 3.0 and pH = 4.5 presented in **Fig.6B**. For example, the droplet size $D_{4,3}$ of the WT1 stabilized emulsion dropped down from 11.1 μm at pH 4.5 back to 0.856 μm at pH 3.0. This was attributed to the fact that while the droplets aggregated at pH 4.5, they did not immediately coalesce. Thus, when a sufficient degree of surface charge was regained at pH 3.0 (ζ-potential = +45.9 ± 1.5 mV, see **Table 1**), the clusters broke down to smaller sizes.

5.2 Emulsions based on conjugated WPI and WPHs samples

When WPI/WPHs were conjugated with maltodextrin, both the emulsifying and stabilizing abilities were enhanced substantially at all tested pH conditions (**Fig.8B**). The difference was particularly pronounced at pH 4.5 where emulsion droplets stabilised by unconjugated WPI/WPHs became strongly aggregated. For example, unlike the highly flocculated state of WT1 stabilized emulsion at pH 4.5, the conjugated WT1 based emulsion sample stayed well dispersed throughout the whole storage period, with no change in droplet size ($D_{4,3}$ was 0.660 μm at day 1 and 0.657 μm at day 60, see **Fig.9B**), although the ζ–potential measured at pH 4.5 was only 1-2 mV (**Table 2**). Nonetheless, there is an increase in droplet size in our emulsions during storage for those samples stabilised by

conjugates formed from the more highly fragmented proteins. For example, the mean droplet size $D_{4,3}$ of fresh emulsion at pH 7.5 was 0.682 μm at day 1 for WT3-MD, but this grew to 1.47 μm after the same storage period of 60 days (**Fig.8B**).

6. The evolution of the mean droplet size, $D_{4,3}$, of emulsions stabilised by SST3, stored at pH 7.5

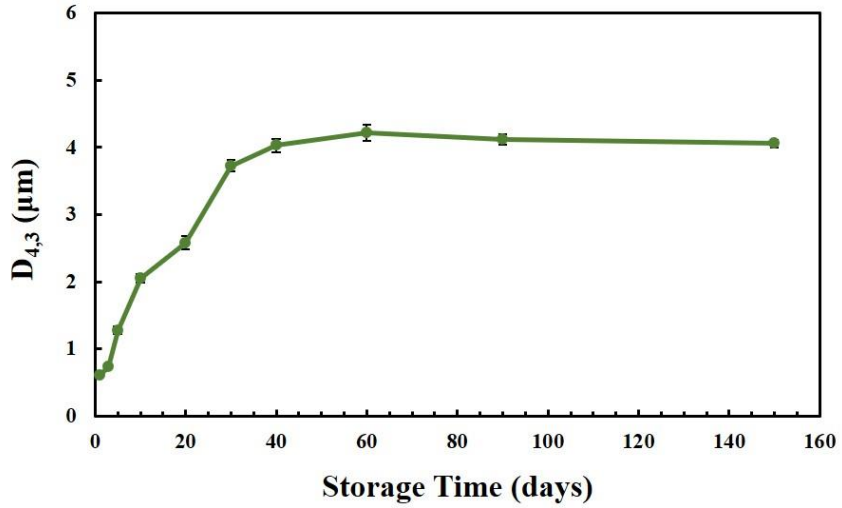

Fig.S5 The change of mean droplet size $D_{4,3}$ for O/W emulsion droplets, stabilised by SST3, stored at pH 7.5.

The emulsion made by SST3 stored at pH 7.5 exhibited a sharp increase in the mean droplet size during the first 30 days. However, the size remained relatively unchanged at around 4 μm after that.

7. The flow behaviour of various emulsion samples stabilised by different emulsifiers

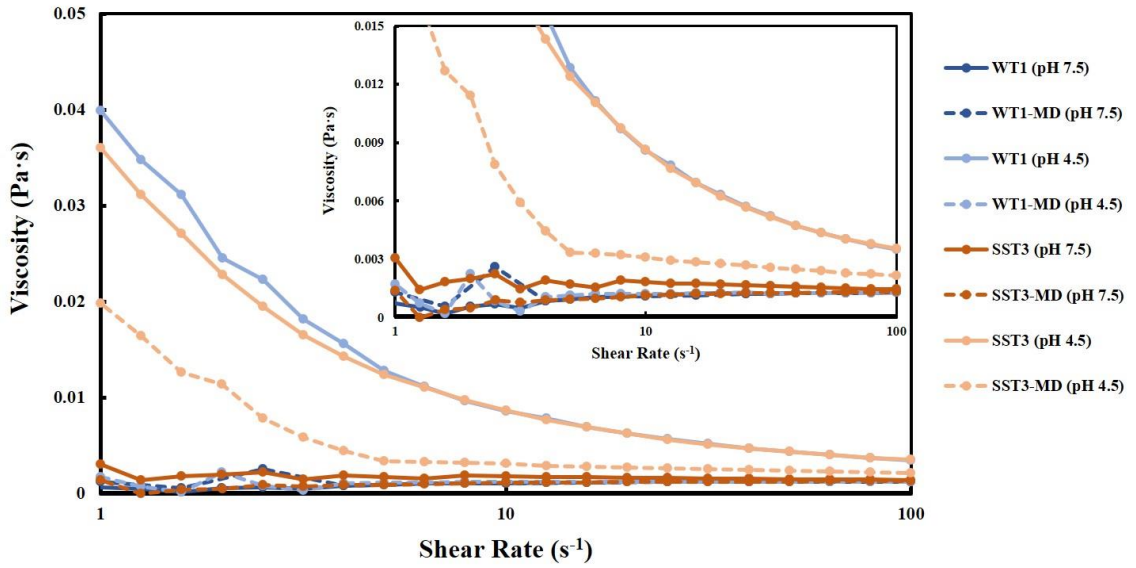

Fig.S6 Apparent viscosity of O/W emulsions made by various emulsifying agents (i.e. WT1 and WT1-MD, SST3 and SST3-MD), stored at pH 7.5 and 4.5 after 60 days of storage, plotted against shear rate. The inset is a magnification of the graphs at low viscosities.

From the above figure, it is seen that all the emulsion samples stored at pH 7.5 exhibited near Newtonian flow behaviour, indicating the absence of flocculated oil droplets in these systems.

The emulsions made from non-conjugated biopolymers (i.e. WT1 and SST3) that were stored at pH 4.5 displayed a dramatic increase in apparent viscosity at low shear rates and a clear shear-thinning type behaviour. This supported our other microscopy and particle size measurement results that these emulsion samples were in a flocculated state. In contrast, the emulsion sample made by conjugated SST3 (i.e. SST3-MD), stored at pH 4.5, displayed significantly smaller low-shear viscosity with an improved flocculation stability (as discussed in section 3.4.2). Nonetheless, the viscosity was still not quite as low as its value at pH=7.5, and some degree of shear thinning is still evidence for this system. In contrast, the flow behaviour of emulsion stabilized by conjugated WT1 (i.e. WT1-MD) stored also at pH 4.5 and pH 7.5 were almost identical. This demonstrates the more superior ability of the conjugated WPH based emulsifier to retain emulsion stability against changes in pH.

8. The coarsening of emulsion sample fabricated by fragmented soy protein SST3

As discussed in section 3.4.1, the coarsening of emulsion cannot simply be attributed to droplet coalescence. Instead, it is tempting then to associate this development of larger droplets taking place over longer periods to Ostwald ripening. The process of Ostwald ripening is fairly insensitive to the type of emulsifier used, but is mainly controlled by the solubility of the dispersed phase in the dispersion medium (McClements, 2015a). For the process to be significant, the oil phase is required to be sufficiently soluble in the aqueous phase, as the phenomenon involves the mass transportation of oil molecules from smaller to larger droplets (Dickinson, 1992; Tcholakova, Denkov, Ivanov, & Campbell, 2006). In our case, sunflower oil was used to prepare emulsions, which is fairly hydrophobic and insoluble in water. Moreover, the emulsion sample made from WT1 stored at pH 7.5 was extremely stable, showing no evidence for Ostwald ripening during the 60 day of storage period (see W-7.5-60 in **Fig.7B**). These facts suggest that the formation of larger droplets in our soy protein hydrolysates stabilised samples is not the result of a straightforward Ostwald ripening process, at least not one driven by the direct migration of oil molecules between the droplets.

At present we have no definitive evidence for the underlying process driving the observed gradual formation of these larger droplets in these systems. Nonetheless, a possibility worth further investigation concerns the presence of soy phospholipids on emulsion stability, as suggested by the observations of Tirok, Scherze, and Muschiolik (2001), Drapala, Auty, Mulvihill, and O' Mahony (2015) and Drapala, Auty, Mulvihill, and O'Mahony (2016). This point is discussed below.

Soy phospholipids are a mixture of low-molecular-weight surfactants and are important constituents of the oil bodies in soybeans (Matsumura, Sirison, Ishi, & Matsumiya, 2017). A typical soy phospholipid residual of 3% (w/w) is often reported in commercial SPI (Arora & Damodaran, 2011; Samoto, et al., 2007). Similar to other kinds of low-molecular-weight surfactants, soy phospholipids facilitate the emulsification process. However, they are not able to guarantee the long-term stability of emulsions (Bos & Van Vliet, 2001; McClements, 2015b). In our fragmented soy protein based emulsions, the residual soy phospholipids (also known as soy lecithin), while only present in small amounts, are nonetheless able to partially displace proteins from the surface of droplets. This could disturb the viscoelastic network of protein films at the oil droplet surface and introduce small patches at the interface that may lack sufficient degree of protection from proteins (Bos, et al., 2001; Petkov,

Gurkov, Campbell, & Borwankar, 2000; Pugnaloni, Dickinson, Ettelaie, Mackie, & Wilde, 2004; Pugnaloni, Ettelaie, & Dickinson, 2003). Furthermore, and perhaps more significantly, it is known that nonpolar molecules can be transported between dispersed oil droplets via solubilisation in surfactant micelles (McClements, 2015b; Moulik, 1996). Although this is still a slow mode of transportation for oil molecules through the aqueous phase, it nonetheless allows for Ostwald ripening process to proceed at a somewhat faster rate than otherwise in the absence of soy phospholipids.

Irrespective of the actual mechanisms responsible for the formation of the large oil drops, we have found experimental evidence that soy phospholipids can accelerate the growth of emulsion droplets during storage. A particularly clear example of this phenomenon occurs in the otherwise very stable hydrolysed milk whey protein stabilized emulsions, upon addition of soy phospholipids. We were able to confirm this result by spiking the WT1-stabilised emulsion (found otherwise to be stable at pH 7.5 for over 60 days) with a small amount of soy lecithin (i.e. 3 g lecithin/100 g WT1).

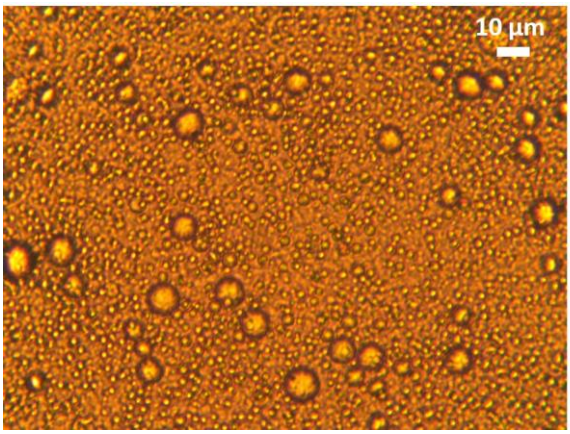

Fig.S7 Micrograph of stored WT1 based emulsion at pH 7.5, with addition of 0.03% soy lecithin (i.e. 3 g soy lecithin/100 g WT1), following 60 days of storage.

The above figure **(Fig.S7)** shows the microstructure of stored WT1 based emulsion at pH 7.5 with addition of soy lecithin. The O/W emulsion was made according to the recipe in section 2.8, except that the oil phase was added with 0.03% (w/w) soy lecithin (i.e. 3 g/100g WT1 sample). The lecithin was gently dissolved in oil by stirring for 2 h. The amount of lecithin added was chosen to be typical of the residual lecithin found in commercial SPI.

This experiment was carried out to assess the impact of this surface active impurity on the storage behaviour of a known stable emulsion at pH = 7.5. After 60 days of storage, the micrograph here taken for this otherwise very stable WT1 stabilized emulsion, looked remarkably similar to one obtained for fragmented soy protein stabilised emulsion (see S-7.5-60 in **Fig.7A**). Therefore, the inferior long term stability of emulsions fabricated by soy protein fragments, when compared to WPI, may well be due to the presence of the lecithin impurity, rather than any inherent properties of these polypeptides per se.

One may speculate then that if the commercial isolated soy protein used here was further purified from the residual lecithin, the storage stability for (trypsin produced) SSPHs based emulsions may have been just as impressive as those obtained with WPI or its low DH hydrolysates. The exact role played by soy phospholipids in causing the formation of large droplets and the presence of Ostwald ripening in our system are suggested possibilities (but not totally unreasonable ones in light of results in **Fig.S7**). However, a more definitive conclusion, will require more careful experimental verification in future, which is outside the scope of the present study.

9. The microstructure of emulsion samples fabricated using SST3 fragments conjugated with maltodextrin (DE4-7) and dextran

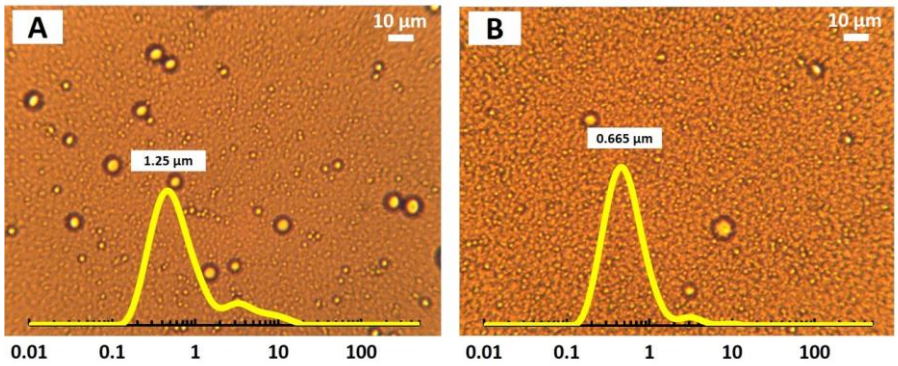

Fig.S8 Micrographs of emulsions stored at pH 7.5 after 60 days. The emulsions were stabilized by conjugated SST3 + maltodextrin DE4-7 (M_w= 65 kDa) and SST3 + dextran (M_w = 500 kDa), which are displayed in (A) and (B), respectively. The droplet size distribution and the mean droplet size $D_{4,3}$ are also superimposed on each photo.

We prepared conjugated SST3 with maltodextrin DE4-7 (MD7, M_w= 65 kDa) and dextran (DX, M_w= 500 kDa), according the recipe in section 2.8. It was observed that the emulsion stability at pH 7.5 progressively improved with an increase in M_w of the polysaccharide. For the conjugated soy peptides with polysaccharides of the highest M_w used here, the emulsions remained reasonably stable following 60 days of storage, retaining $D_{4,3}$ = 0.665 µm as compared to 0.598 µm measured on the first day.

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